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# Seedling development n Stylisma pickeringii var. pattersonii (Convolvulaceae), an endangered Illinois species

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SEEDLING DEVELOPMENT IN STYLISMA PICKERINGII VAR. PATTERSONII

(CONVOLVULACEAE), AN ENDANGERED ILLINOIS SPECIES

(TITLE)

BY

Jason B Cunningham

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# SEEDLING DEVELOPMENT IN *STYLISMA PICKERINGII* VAR. *PATTERSONII* (CONVOLVULACEAE), AN ENDANGERED ILLINOIS SPECIES

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## ABSTRACT

*Stylisma pickeringii* (Torr.) Gray var. *pattersonii* (Fern. & Schub.) Myint (Convolvulaceae) is listed as an endangered plant species in Illinois and is commonly known as Patterson bindweed or Patterson dawnflower. It is a prostrate vine that occurs in well-drained sand prairies and sandy open woods. High temperature, low moisture holding ability and blowing sand in these prairies create a harsh environment for seedling establishment.

Seedlings of *Stylisma pickeringii* show a unique pattern of development, which may enhance their ability to become established. Following radicle and cotyledon emergence, an enlarged ring encircling the radicle, referred to as the transition zone, develops. A shoot arises laterally from this hypocotyl region, which is situated below the soil surface. All further growth above the soil surface continues from this region. Previous studies indicated that light is involved with

seedling development, but it is unclear whether photoperiod or total irradiance, or both are influencing this process.

The first objective of this study was to determine the effect of photoperiod and light intensity on seedling development of *Stylisma pickeringii*, especially the effect on the lateral shoot development. The second objective of this study was to document the anatomical development of *S. pickeringii* seedlings, as well as determine the origin of the lateral shoots.

For light studies, plants were grown from seed both in Promix:Sand (1:1) and *in vitro* on Murashige and Skoog medium with long days (16 hours light/ 8 hours dark) and short days (8 hours light/ 16 hours dark) at two light intensities (700 and 350 μmol $s^{-1}m^{-2}$). Development of radicles, cotyledons and shoots was measured to determine the effect of light on seedling development. Radicles and cotyledons emerged sooner with higher light intensity than with lower light intensity, whereas photoperiod had no significant effect. However, shoot emergence was earlier with long days than short days. With short days, shoots developed faster at the higher light intensity than at the lower light intensity. Overall development of seedlings, including the unique shoot development, was more controlled by total irradiance than by photoperiod.

For anatomical studies, plants were grown from seed *in vitro* on Murashige and Skoog medium. Seedlings were removed from tubes and placed in FAA at 5 day

intervals until day 35. Tissues were embedded in Paraplast and sectioned at 10 µm both longitudinally and transversely. Permanent slides were made using the Safranin O and Fast Green staining procedure. Development of the meristematic region, resembling a shoot apical meristem, occurs basally in a cavity created by the fusion of cotyledonary petioles. Once the developing shoot is too large to be contained within the cavity, it becomes outwardly visible as it tears through the fused petioles that encircle it.

This adaptation enables the young shoot to mature within the protected sheath before it emerges into the harsh sand prairie environment. While the lateral shoot is a distinctive characteristic of *Stylisma pickeringii* seedlings, the fused cotyledonary petioles and its sunken shoot apical meristem may be the most significant factors enhancing seedling establishment.

## ACKNOWLEDGEMENTS

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# CHAPTER 1:

## Introduction

*Stylisma pickeringii* (Torr.) Gray var. *pattersonii* (Fern. & Schub.) Myint (Convolvulaceae) is listed as an endangered species in Illinois and is commonly known as Patterson bindweed or Patterson dawnflower. A prostrate vine, it occurs in well-drained sand prairies and sandy open woods. This perennial plant grows with multiple stems bearing linear leaves with entire margins. In Illinois, only five known populations occur in three counties of western Illinois, all restricted to the sand prairies along the Illinois and Mississippi Rivers (Herkert and Ebinger, 2002).

Two varieties of *Stylisma pickeringii* are recognized: var. *pickeringii* and var. *pattersonii*. *S. pickeringii* var. *pickeringii* has longer stylar branches and obtuse sepals, whereas *S. pickeringii* var. *pattersonii* has shorter stylar branches and acute sepals (Myint, 1966). Populations of *Stylisma pickeringii* var. *pickeringii* occur in the southern and eastern United States (Myint, 1966; USDA, NRCS, 2004). *Stylisma pickeringii* var. *pattersonii* is distributed throughout the south-central U.S., with the populations in Illinois being on the northeastern extreme of its range (Herkert and Ebinger, 2002; USDA, NRCS, 2004). *Stylisma pickeringii* var. *pattersonii* will be the focus of the following studies.

*Stylisma pickeringii* var. *pattersonii* flowers from June to September, producing a mass of white flowers resembling those of the more common morning glory also in the Convolvulaceae. Peak flowering occurs in July, when the plant is visited frequently by *Apis mellifera*, *Heterostylum croceum* and other Bombyliidae (Todd, Owen, Coons and Webb, 2002). Fruits of *S. pickeringii* are usually 1- or 2-seeded (Myint, 1966; Todd, 2002). Seed is produced profusely in the field and is released from the plant during late summer. Seeds accumulate in the uppermost 5.0 cm of the soil surface beneath the plant (Claerbout, 2003). A mechanism for seed dispersal has not yet been identified. A lack of seed dispersal may be one cause for the limited distribution of *Stylisma pickeringii* in Illinois. Seeds are produced in three distinct colors (yellow, tan and maroon). Yellow seeds are the most vigorous, having higher germination rates and more healthy internal appearance than the other seed colors (Todd, 2002). Seeds have a demonstrated dormancy that may be broken by soaking the seeds for 120 minutes in concentrated $H_2SO_4$ (Todd, Coons and Owen, 2002). Soaking seeds in a 15% bleach solution for 20 minutes and then rinsing them in distilled water will surface disinfest them in preparation for tissue culture. Seeds also may be stored for at least one year without significantly decreasing their viability (Todd, 2000).

Seedlings of *Stylisma pickeringii* express a unique pattern of development. The radicle emerges from the seed with germination at 5 days after planting, and cotyledons occur above the ground at 22 days after planting. A thickened region

develops, forming a ring around what appears to be the hypocotyl region, approximately 14 mm below the media surface in tissue culture at 25 days after planting (Claerbout, 2003). This region appears to be the transition from root to shoot in the seedling. A lateral shoot then develops and emerges from that transition zone 7.2 cm below the surface of the soil in the field (Todd, 2002). The cotyledons become necrotic and the above ground growth continues when this lateral shoot elongates and emerges through the soil surface.

Factors that may affect the development of the lateral shoot were studied previously, including carbohydrate availability, light and photoperiod. These studies are not, however, in agreement concerning the effect of photoperiod on lateral shoot development. Claerbout (2003) indicated that a higher percentage of plants develop a lateral shoot with short day than long day conditions in a greenhouse study. A study conducted in tissue culture indicated that a higher percentage of plants develop lateral shoots with long day conditions (Kerber et al., 2000). Lateral shoot initiation occurs only when seedlings are exposed to light. Carbohydrate availability alone, however, does not significantly affect lateral shoot development (Kerber et al., 2003).

Protocols for growing *Stylisma pickeringii* seedlings in greenhouses and growth chambers also were established through several studies conducted at Eastern Illinois University since 1999. Growing medium for optimal seedling establishment of *S. pickeringii* is 3:1 greenhouse mix:torpedo sand compared to

100 percent torpedo sand and 3:1 torpedo sand: greenhouse mix (DuFrain, 1999). Media containing 2 g/L Gelrite, 4.3 g/L Murashige and Skoog salts (JRH Biosciences, Lenexa, KS), 0.1 g/L myoinositol and 30 g/L sucrose encourages seedling establishment in tissue culture (Claerbout, 2003). Seedlings grown in the greenhouse also remained alive when watered daily with 30 ml of distilled water compared to 30 ml once a week or 60 ml once a day, twice a week and once a week (Heisler et al., 1999).

The overall goal of this research was to examine the development of the lateral shoot in *Stylisma pickeringii* var. *pattersonii*. This goal was achieved by:

1) studying the effect of both photoperiod and light intensity on the development of *Stylisma pickeringii* var. *pattersonii* seedlings grown in both tissue culture and soilless mix, specifically the lateral shoots, and
2) examining the anatomy of *Stylisma pickeringii* var. *pattersonii* seedlings at distinct stages in development focusing on the origin of the lateral shoot.

This research will expand the understanding of *Stylisma pickeringii* var. *pattersonii* relative to its pattern of seedling establishment and how its adaptations relate to its survival in the sand prairie.

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# CHAPTER 2:

## Seedling development of *Stylisma pickeringii* as affected by photoperiod and light intensity


### ABSTRACT

*Stylisma pickeringii* var. *pattersonii* is an Illinois endangered sand prairie species with unique seedling development where lateral shoots arise from tissue that is below the soil surface and appears to be the hypocotyl. Previous studies indicate that light is involved with initiation and development of these lateral shoots, but it is unclear whether photoperiod or total irradiance is more important. The objective of this study was to determine the effect of photoperiod and light intensity on seedling development of *S. pickeringii*. Plants were grown from seed both in Promix:sand (1:1) and *in vitro* on Murashige and Skoog medium with long days (16 hours light/ 8 hours dark) and short days (8 hours light/ 16 hours dark) at two light intensities (700 and 350 µmol $s^{-1}m^{-2}$). Development of radicle, cotyledons, and lateral shoots was measured. In plants grown *in vitro,* radicles, transition zones and cotyledons emerged sooner with higher light intensity than with lower light intensity, whereas photoperiod had no significant effect. However, shoot emergence was earlier with long days than short days. With short days, shoots of plants emerged sooner at the higher light intensity than at the lower light intensity. Although lateral shoots emerged sooner with higher levels of irradiance, growth rates of lateral shoots were higher for plants grown with short day conditions and low light intensity than those grown with long day conditions and high light intensity. In plants grown in Promix and sand, neither

final lateral shoot length nor relative growth rate of lateral shoots were significantly affected by light intensity or day length. Overall, the development of seedlings, including the unique shoot development, was more controlled by total irradiance than by photoperiod.

## INTRODUCTION

*Stylisma pickeringii* (Torr.) Gray var. *pattersonii* (Fern. & Schub.) Myint only occurs in three counties of Illinois (Cass, Henderson and Mason), and is listed as an endangered species in the state (Herkert and Ebinger, 2002). *S. pickeringii*, commonly known as Patterson bindweed, is a perennial, prostrate vine that occurs in well-drained sand prairies and sandy open woods along the Mississippi and Illinois Rivers. Sand habitats frequently have low moisture, shifting sands, and high light intensities that make seedling establishment difficult (Bach, 1998).

*Stylisma pickeringii* seedlings possess an unusual morphology that may serve as an adaptation to the harsh environment (Myint, 1966; Todd, 2002; Claerbout, 2003). Following seed germination and emergence of the radicle and cotyledons, a shoot arises laterally from an apparent hypocotyl region, which is situated below ground. The lateral shoot originates an average of 7.25 cm below the soil surface in the field, but only 1.5- 2.0 cm below the surface of the medium in tissue culture (Todd, 2002; Claerbout, 2003). All post-cotyledonary growth above the soil surface originates from this subterranean region, in contrast to typical seedling development where the apical meristem is positioned between the cotyledons (Todd, 2002). Its endangered status, coupled with an atypical pattern of seedling development, has prompted several studies of this species (Kerber et al., 2000; Claerbout, 2003; Kerber et al., 2003).

*Stylisma pickeringii* seedlings grown *in vitro* demonstrated that, with a long day photoperiod, plants had significantly greater lateral shoot initiation and lateral shoot length than with a short day photoperiod (Kerber et al., 2003). Another study, conducted in a greenhouse, indicated that lateral shoot initiation and lateral shoot length were greater with a short day photoperiod than with a long day photoperiod (Claerbout, 2003). Seedlings grown with short day conditions in the latter study were exposed to a higher light intensity (434 $\mu$mol $s^{-1}$ $m^{-2}$), than those with long day conditions (252 $\mu$mol $s^{-1}$ $m^{-2}$), which may contribute to these conflicting results.

While previous studies indicate that light is involved with seedling development, it remains unclear whether photoperiod or total irradiance is more important. The objective of this study was to determine the effect of photoperiod and light intensity on seedling development of *S. pickeringii*, specifically their effect on lateral shoot development.

## MATERIALS AND METHODS

**Plant material:**

*Sylisma pickeringii* var. *pattersonii* seeds collected 19 September 2002 near Snicarte (Mason Co.), Illinois were scarified with concentrated sulfuric acid (18 M $H_2SO_4$) for two hours. Seeds were removed and rinsed in distilled water for five minutes. Seeds were planted in either tubes with tissue culture medium or in containers with soilless medium.

**Culture environment:**

Plants were grown in a growth chamber (Conviron CMP 4030, Winnipeg, Manitoba, Canada) at 25.0 ± 1.0°C. Four light treatments were used: 1) short day (8 hours light, 16 hours dark) with low light intensity (350 $\mu$mol $s^{-1}m^{-2}$), 2) short day (8 hours light, 16 hours dark) with high light intensity (700 $\mu$mol $s^{-1}m^{-2}$), 3) long day (16 hours light, 8 hours dark) with low light intensity (350 $\mu$mol $s^{-1}$ $m^{-2}$), and 4) long day (16 hours light, 8 hours dark) with high light intensity (700 $\mu$mol $s^{-1}m^{-2}$). Light levels were measured every other week during the study using a LI-185A photometer (Li-Cor, Lincoln, NE).

**Seedlings in culture tubes:**

Four hundred scarified seeds were surface sterilized by placing them in a 20% bleach solution (7% NaOCl, Homebest UltraBleach) for 20 minutes. Then seeds were removed and rinsed in distilled water for 10 minutes. Seeds were planted in 25 x 150 mm culture tubes containing 20.0 mL of sterile media containing 4.3 g/L Murashige and Skoog salts (JRH Biosciences, Lenexa, KS), 0.1 g/L myoinositol, 30 g/L sucrose and 8 g/L agar (Murashige and Skoog, 1962). Seeds

were placed approximately 3 mm into the agar with the blunt end of the seed oriented downward. One hundred tubes were used in each of the four light treatments in the growth chambers. All tubes were observed daily for 70 days. Days of radicle, transition zone, cotyledons, and lateral shoot emergence were recorded. Lengths of lateral shoots also were recorded on days 40, 50, 60 and 70.

**Seedlings in soilless mix and sand:**

Four hundred scarified seeds were dusted with the fungicide Thiram (50% tetramethylthiuram disulfide, Loveland Industries, Cambridge, Cambridgeshire, UK). Seeds were planted 2.5 cm deep in Rootrainer® books (Hummert, St. Louis, MO) containing Promix:sand (1:1). Rootrainer® books are cells that may be opened by unfolding them to extract the seedling with little damage to the roots and other below ground structures. Seedlings were watered daily using distilled water, and fertilized every other week with Peter's® 20-20-20 (The Scotts Company, Marysville, OH) at 250 mg fertilizer per liter water. One hundred seeds were used in each of the four light treatments. Ten seedlings were removed every week when the presence of a radicle, transition zone, lateral shoot and leaves on the lateral shoot was recorded. Root length (distance from root tip to soil surface), lateral shoot length, and distance from lateral shoot to the soil surface also were recorded weekly for ten weeks.

**Data analyses:**

Relative growth rates of lateral shoots for plants grown in tissue culture and soilless mix were calculated as ($\ell$n {lateral shoot length$_{t2}$} –$\ell$n {lateral shoot

$length_{t1}\}) / (t_2-t_1)$, using the four measurements at days 40, 50, 60 and 70, which represented the linear portion of the data and the time when lateral shoots were present. Data analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL) to calculate two-way ANOVAs. Means are expressed plus or minus standard errors.

## RESULTS

For *Stylisma pickeringii* var. *pattersonii* seedlings grown *in vitro*, radicles, transition zones and cotyledons developed significantly sooner with high light intensity than with low light intensity ($p = 0.021$, 0.022, and 0.002, respectively) for both short and long days (Tables 1 and 2). Photoperiod, however, did not have a significant effect on radicle, transition zone or cotyledon development ($P > 0.05$). A significant interaction was found between day length and light intensity for lateral shoot emergence ($p = 0.032$). Lateral shoot emergence occurred earlier in long day than short day photoperiods. However, with short day conditions, lateral shoots of plants emerged faster at the high light intensity than at the low light intensity (Table 1). Emergence of lateral shoots grown with long days was not affected by light intensity (Table 2). The lateral shoot lengths for seedlings with long days and 350 $\mu$mol $s^{-1}$ $m^{-2}$ was greatest on all days (40, 50, 60 and 70) when compared to all other treatments (Figure 1). At least 50% of *S. pickeringii* seedlings grown *in vitro* in all treatments remained uncontaminated and reached maturity (cotyledons emerged and plants developed a lateral shoot) during the duration of the study (Table 3).

*Stylisma pickeringii* seedlings grown *in vitro* had significantly ($p = 0.032$) greater relative growth rates with short days than long days (Figure 2). Relative growth rates also were significantly ($p < 0.001$) greater for lateral shoots of seedlings grown with low than high light intensities (Figure 2).

Radicles of *Stylisma pickeringii* var. *pattersoni* seedlings grown in soilless mix and sand reached their maximum length, approximately 10 cm, only two weeks after planting for all treatments. Nearly all seedlings had developed roots within the first week regardless of treatment (Table 4). Final radicle lengths were not significantly affected by light intensity or day length ($p = 0.246$ and 0.122 respectively) (Figure 3). One plant had a raised ring in the hypocotyl region, referred to as the transition zone, after one week of development in the short day with low light intensity. The presence of a transition zone also was detected in plants grown with long day conditions, both in the high and low light intensities, only two weeks after planting. However most plants possessed a transition zone by the third week (Table 5). Lateral shoots developed in seedlings grown in soilless mix and sand for all treatments by three weeks after planting (Table 6). Final lateral shoot lengths were not significantly affected by light intensity or day length ($p = 0.763$ and 0.053 respectively) (Figure 4). The lateral shoots did not develop leaves until they reached the soil surface and were exposed to light. By week 6 most of the lateral shoots had emerged from the soil surface and leaves were conspicuous (Table 7).

The relative growth rate of lateral shoots for *Stylisma pickeringii* seedlings grown in soilless mix and sand was not significantly affected by either light intensity or day length ($p = 0.881$ and 0.386 respectively) (Figure 5).

## DISCUSSION

Development of *Stylisma pickeringii* var. *pattersonii* seedlings, including development of the lateral shoot, is more controlled by total irradiance than by photoperiod. Emergence of radicle, transition zone and cotyledons occurred earlier with high light intensity than with low light intensity, whereas photoperiod did not have a significant effect. Once the lateral shoot emerges, however, relative growth rates were greatest with short days and lower light intensities. These findings suggest that, although exposure to more light enhances the speed at which the lateral shoot emerges, it may be detrimental to the development of the young lateral shoot after emergence. High light intensities (>1000 $\mu$mol $s^{-1}$ $m^{-2}$) in the natural habitat of *S. pickeringii* would suggest that the plant should thrive with the higher light intensity (700 $\mu$mol $s^{-1}$ $m^{-2}$) used in this study. However, plants grown *in vitro* often are not well adapted to prevent water loss. Functioning stomates and well-developed cuticles that plants in the field possess do not develop with *in vitro* plants, as they are exposed to high humidity levels in the culture tube (Trigiano and Gray, 2000). If light intensities caused the temperature to increase within the culture tube, evaporative losses may have also increased for seedlings. Increased evaporative losses would adversely affect cell enlargement and hence decrease the relative growth rate of the lateral shoot.

Characteristics of *Stylisma pickeringii* seedlings grown in tissue culture may differ from those grown in soil. No *in vitro* seedlings examined in this study had more than one lateral shoot emerging from the transition zone. However, field observations (Todd, 2002) and other studies conducted in the lab (pers. obs.), where *S. pickeringii* seedlings were grown in soil, demonstrated that several shoots may emerge from a single transition zone. *Stylisma pickeringii* seedlings grown *in vitro* with varied levels of cytokinin exhibited more lateral shoot proliferation in media containing higher levels of cytokinin (Donnelly et al., 2001). Lower levels of cytokinin in plants typically cause stunted shoots and smaller shoot apical meristems (Werner et al., 2001). The portion of the plant from which the lateral shoot originates typically occurs below the soil surface, however, in tissue culture this area is exposed to light. The exposure to light may decrease cytokinin levels, thereby decreasing lateral shoot proliferation (Benková et al., 1999).

No significant effects were observed with *Stylisma pickeringii* seedlings grown in soilless mix and sand. The small, weekly sample size may have been too small to detect any significant differences because growth of seedlings in soilless mix and sand was quite variable. Similar experiments in the future should significantly increase the sample size to account for this variation.

Lateral shoot initiation was greater with long days than short days. With short days, however, plants grown with higher light intensities initiated lateral shoots

earlier than those in lower light intensities. These findings contradict findings of Claerbout (2003) in which lateral shoot initiation was greater with short days than with long days. However, these results are in agreement with previous experiments conducted in tissue culture by Kerber *et al.* (2003). The conflicting results in Claerbout (2003) may be due to the short day plants being exposed to a higher light intensity than the long day plants. The results of this study indicate that lateral shoot initiation is dependent on total irradiance rather than photoperiod as plants developed lateral shoots regardless of photoperiod.

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**Table 1. Days until development of radicle, transition zone, cotyledons, and lateral shoots in *Stylisma pickeringii* var. *pattersonii* grown *in vitro* with short days (8 hours light/ 16 hours dark) at two light intensities (350 and 700 µmol $s^{-1}m^{-2}$).**

| | Days until emergence | |
|---|---|---|
| | 350 | 700 |
| Radicle | 5.8 ± 1.3[z] | 3.7 ± 0.2 *[y] |
| Transition Zone | 10.5 ± 1.0 | 8.4 ± 0.4 * |
| Cotyledons | 16.5 ± 1.1 | 14.0 ± 0.5 * |
| Lateral Shoot | 42.5 ± 1.6 | 36.0 ± 1.9 * |

[z] Means ± standard errors
[y] Means within a row followed by * are significantly different based on a two-way ANOVA at p = 0.05.

**Table 2. Days until development of radicle, transition zone, cotyledons, and lateral shoots in *Stylisma pickeringii* var. *pattersonii* grown *in vitro* with long days (16 hours light/ 8 hours dark) at two light intensities (350 and 700 µmol $s^{-1}m^{-2}$).**

| | Days until emergence | |
|---|---|---|
| | 350 | 700 |
| Radicle | 4.4 ± 0.4[z] | 3.3 ± 0.2 *[y] |
| Transition Zone | 10.4 ± 0.7 | 9.1 ± 0.5 * |
| Cotyledons | 15.2 ± 0.6 | 13.1 ± 0.6 * |
| Lateral Shoot | 31.9 ± 1.0 | 32.1 ± 1.6 |

[z] Means ± standard errors
[y] Means within a row followed by * are significantly different based on a two-way ANOVA at p = 0.05.

**Table 3. Percentage of *Stylisma pickeringii* var. *pattersonii* seedlings grown *in vitro* that became contaminated or developed a lateral shoot by day 70 when grown with short days (8 hours light/ 16 hours dark) or long days (16 hours light/ 8 hours dark) at two light intensities (350 and 700 µmol $s^{-1}m^{-2}$).**

| | Percentage of Seedlings | | |
|---|---|---|---|
| Light Treatment | Contaminated | Not Developing a Lateral Shoot | Developing a Lateral Shoot |
| Short Day 350 | 34 | 15 | 51 |
| Long Day 350 | 37 | 4 | 59 |
| Short Day 700 | 20 | 27 | 53 |
| Long Day 700 | 32 | 8 | 60 |

**Table 4. Percentage of *Stylisma pickeringii* var. *pattersonii* seedlings grown in soilless mix and sand that developed a radicle when grown with short days (8 hours light/ 16 hours dark) or long days (16 hours light/ 8 hours dark) at two light intensities (350 and 700 µmol $s^{-1}m^{-2}$).**

| Week | Short Day 350 | Long Day 350 | Short Day 700 | Long Day 700 |
|---|---|---|---|---|
| 1 | 90 | 90 | 80 | 70 |
| 2 | 90 | 60 | 90 | 90 |
| 3 | 90 | 80 | 50 | 80 |
| 4 | 80 | 80 | 90 | 80 |
| 5 | 100 | 100 | 80 | 100 |
| 6 | 100 | 80 | 70 | 80 |
| 7 | 80 | 90 | 50 | 90 |
| 8 | 100 | 90 | 80 | 50 |
| 9 | 90 | 80 | 90 | 80 |
| 10 | 70 | 90 | 70 | 70 |
| Range | 70 – 100 | 80 – 100 | 50 – 90 | 50 – 100 |

**Table 5. Percentage of *Stylisma pickeringii* var. *pattersonii* seedlings grown in soilless mix and sand that developed a transition zone[z] when grown with short days (8 hours light/ 16 hours dark) or long days (16 hours light/ 8 hours dark) at two light intensities (350 and 700 µmol $s^{-1}m^{-2}$).**

| Week | Short Day 350 | Long Day 350 | Short Day 700 | Long Day 700 |
|---|---|---|---|---|
| 1 | 10 | 0 | 0 | 0 |
| 2 | 0 | 30 | 0 | 40 |
| 3 | 30 | 60 | 50 | 70 |
| 4 | 70 | 70 | 90 | 70 |
| 5 | 100 | 100 | 80 | 100 |
| 6 | 100 | 80 | 80 | 80 |
| 7 | 80 | 90 | 50 | 90 |
| 8 | 100 | 80 | 80 | 50 |
| 9 | 90 | 80 | 80 | 60 |
| 10 | 60 | 90 | 70 | 60 |
| Range | 0 – 100 | 0 – 100 | 0 – 90 | 0 – 100 |

[z] Thickened region from which the lateral shoot emerges below the soil surface

**Table 6.** **Percentage of *Stylisma pickeringii* var. *pattersonii* seedlings grown in soilless mix and sand that developed a lateral shoot when grown with short days (8 hours light/ 16 hours dark) or long days (16 hours light/ 8 hours dark) at two light intensities (350 and 700 µmol $s^{-1}m^{-2}$).**

| Week | Short Day 350 | Long Day 350 | Short Day 700 | Long Day 700 |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 |
| 3 | 10 | 40 | 50 | 70 |
| 4 | 70 | 70 | 90 | 70 |
| 5 | 100 | 90 | 80 | 100 |
| 6 | 90 | 80 | 70 | 80 |
| 7 | 70 | 90 | 50 | 90 |
| 8 | 100 | 80 | 80 | 50 |
| 9 | 90 | 80 | 80 | 50 |
| 10 | 60 | 90 | 70 | 60 |
| Range | 0 – 100 | 0 – 90 | 0 – 90 | 0 – 100 |

**Table 7.** **Percentage of *Stylisma pickeringii* var. *pattersonii* seedlings grown in soilless mix and sand that developed leaves on their lateral shoots when grown with short days (8 hours light/ 16 hours dark) or long days (16 hours light/ 8 hours dark) at two light intensities (350 and 700 µmol $s^{-1}m^{-2}$).**

| Week | Short Day 350 | Long Day 350 | Short Day 700 | Long Day 700 |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 |
| 4 | 0 | 30 | 0 | 40 |
| 5 | 70 | 10 | 70 | 80 |
| 6 | 90 | 50 | 70 | 70 |
| 7 | 70 | 90 | 50 | 90 |
| 8 | 90 | 80 | 70 | 50 |
| 9 | 90 | 70 | 70 | 50 |
| 10 | 50 | 90 | 70 | 60 |
| Range | 0 – 90 | 0 – 90 | 0 – 70 | 0 – 90 |

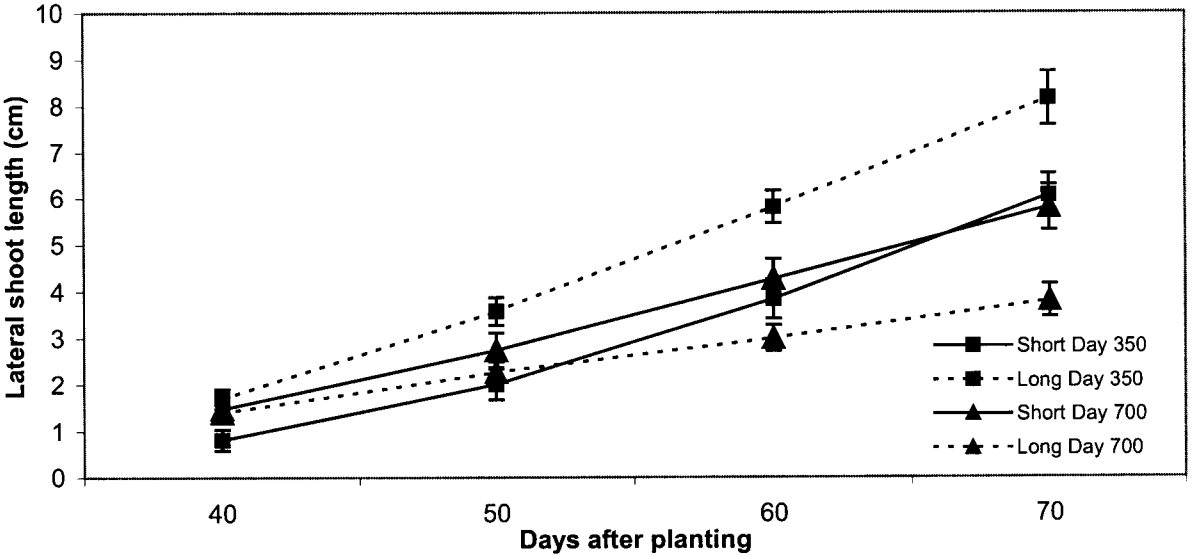


**Figure 1. Lateral shoot length of *Stylisma pickeringii* var. *pattersonii* seedlings grown *in vitro* at 40, 50, 60 and 70 days after planting for short (8 hrs light) and long (16 hrs light) days at two light intensities (350 and 700 umol $s^{-1}$ $m^{-2}$). Mean ± SE**

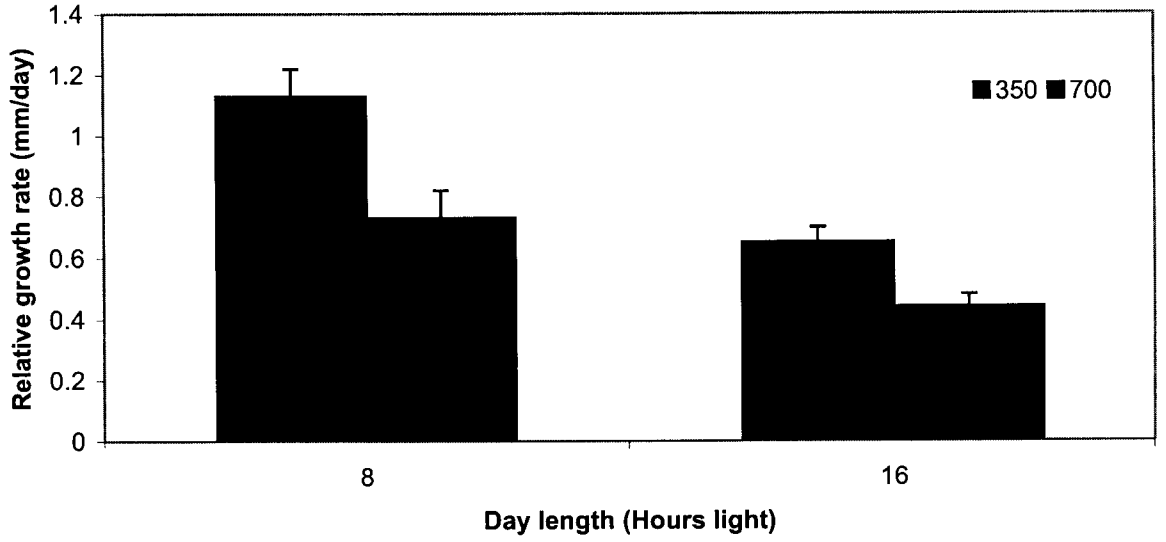


**Figure 2. Relative growth rates of lateral shoots for *Stylisma pickeringii* var. *pattersonii* seedlings *in vitro* with short (8 hrs light) and long (16 hrs light) days at two light intensities (350 and 700 umol $s^{-1}$ $m^{-2}$). Mean ± SE**

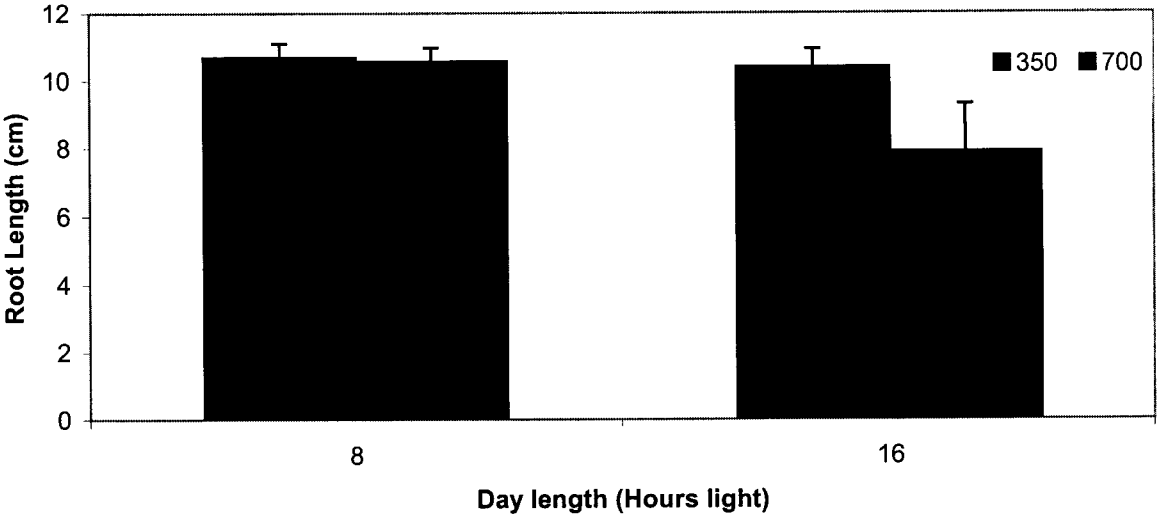


**Figure 3. *Stylisma pickeringii* var. *pattersonii* root length 10 weeks after planting in soilless mix: sand for short (8 hrs light) and long (16 hrs light) days at two light intensities (350 and 700 umol $s^{-1}$ $m^{-2}$). Mean ± SE**

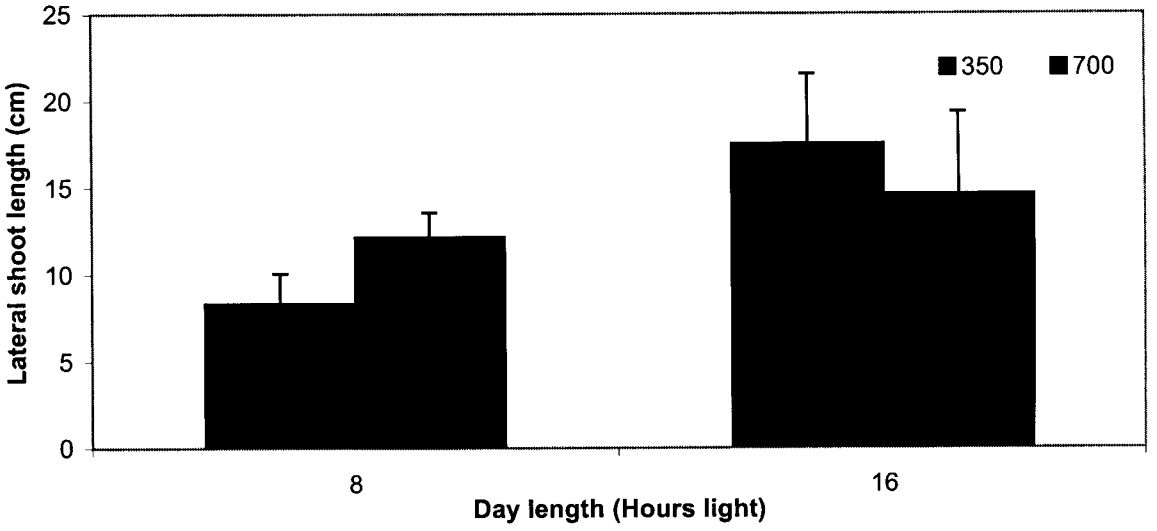


**Figure 4. Length of *Stylisma pickeringii* var. *pattersonii* lateral shoots 10 weeks after planting in soilless mix: sand for short (8 hrs light) and long (16 hrs light) days at two light intensities (350 and 700 umol $s^{-1}$ $m^{-2}$). Mean ± SE**

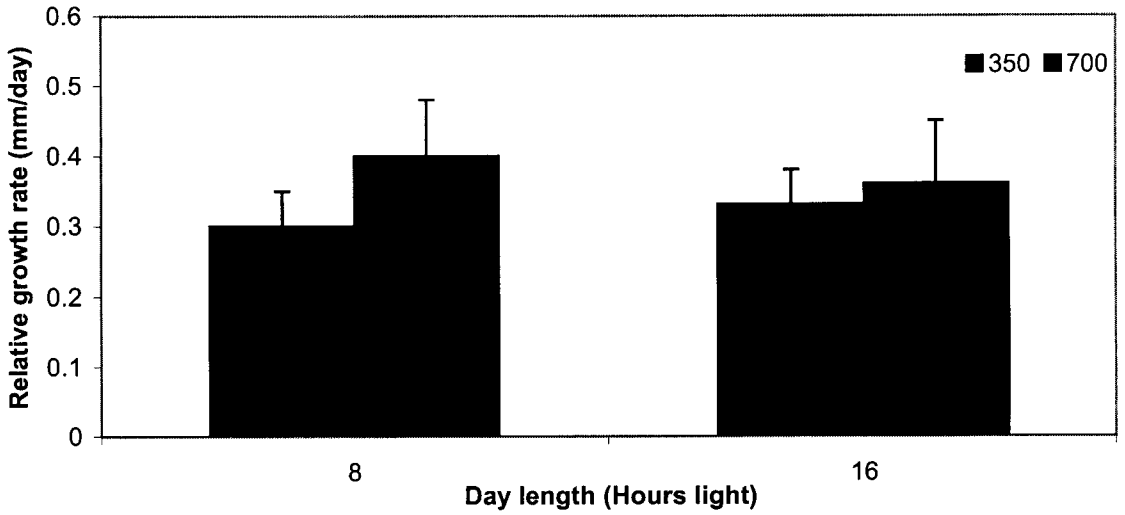


**Figure 5. Relative growth rates of lateral shoots for *Stylisma pickeringii* var. *pattersonii* seedlings in soilless mix: sand with short (8 hrs light) and long (16 hrs light) days at two light intensities (350 and 700 umol $s^{-1}$ $m^{-2}$). Mean ± SE**

# CHAPTER 3:

## Anatomical and morphological study of *Stylisma pickeringii* seedling development


### ABSTRACT

*Stylisma pickeringii* var. *pattersonii* is an Illinois endangered species from sand prairies where high levels of light, low water in surface layer, high temperature and blowing sand create a harsh environment for seedling establishment. Seedling development is unique as a shoot arises laterally from an apparent hypocotyl region that is subterranean. All growth above the soil surface continues from this region. The objective of this study was to determine the origin and anatomy of these lateral shoots. Plants were grown from seed *in vitro* on Murashige and Skoog medium. Seedlings were removed from tubes and placed in FAA at 5 day intervals until day 35. Tissues were embedded in Paraplast and sectioned at 10 µm both longitudinally and transversely. Permanent slides were made using the Safranin O and Fast Green staining procedure. Development of the meristematic region, resembling a shoot apical meristem, occurs near the base of a cavity created by the fusion of cotyledonary petioles. This meristematic region was detectable as early as one day after the emergence of the radicle. The presence of a meristem within a cavity at this early stage in seedling development appears unique to *S. pickeringii*. This adaptation may enhance the ability of the seedling to become established by providing protection for the meristem from the harsh environment where it occurs.

## INTRODUCTION

*Stylisma pickeringii* (Torr.) Gray var. *pattersonii* (Fern. & Schub.) Myint, commonly known as Patterson bindweed, is a perennial, prostrate vine that occurs in well-drained sand prairies and sandy open woods. It is distributed from Iowa to Texas; however, it only occurs in five small populations located within three counties in Illinois, its northeastern most range, where it is listed as an endangered species (Herkert and Ebinger, 2002; USDA, NRCS, 2004). Sand prairies commonly have blowing sand, low water levels in the soil, high temperatures and high light that make seedling establishment difficult (Bach, 1998).

*Stylisma pickeringii* seedlings have a unique developmental pattern (Myint, 1966). Typically, the apical meristem of a seedling, responsible for the elongation of the stem, is exposed above ground and is positioned between the cotyledons (Steeves and Sussex, 1972; Esau, 1977). In *S. pickeringii* however, seed germinates approximately 5 days after planting and cotyledons emerge from the seed coat 22 days after the seed is planted on tissue culture medium. An enlarged ring encircling the hypocotyl, referred to as the transition zone, develops 25 days after planting. An elongated shoot arises laterally from this apparent hypocotyl region, which is situated below the soil surface (Claerbout, 2003). The lateral shoot originates an average of 7.25 cm below the soil surface in the field, however, in tissue culture the lateral shoot occurs nearer the surface of the media at approximately 1.5 - 2.0 cm below the surface (Todd, 2002;

Claerbout, 2003). All growth above the soil surface, including multiple stems, continues from this region. This atypical pattern of seedling development was not found reported in any other species.

Although the unusual pattern of seedling development for the endangered species *Stylisma pickeringii* var. *pattersonii* was studied at the macroscopic level, examination of the anatomy for seedling development at the microscopic level has not been studied (Claerbout, 2003). The objectives of this study were to document the anatomical development of *S. pickeringii* seedlings and to determine the origin of the lateral shoots.

## MATERIALS AND METHODS

### Plant material and growth conditions:

*Sylisma pickeringii* var. *pattersonii* seeds collected 19 September 2002 near Snicarte (Mason Co.), Illinois were scarified with concentrated sulfuric acid (18.0 M $H_2SO_4$) for two hours. Seeds were removed and rinsed in distilled water for five minutes. Surface sterilization was performed by placing them in a 20% bleach solution (7% NaOCl, Homebest UltraBleach) for 20 minutes. Seeds then were removed and rinsed in distilled water for 10 minutes. Seeds were planted in 25 x 150 mm culture tubes on 20.0 mL sterile media containing 4.3 g/L Murashige and Skoog salts (JRH Biosciences, Lenexa, KS), 0.1 g/L myoinositol, 30 g/L sucrose and 8 g/L agar. Seeds were placed approximately 3 mm into the medium with the blunt end of the seed oriented downward. Tubes then were placed in a germination chamber (Percival Scientific, Perry, IA) that maintained constant light (45 $\mu$mol $s^{-1}$ $m^{-2}$) and temperature (25.0 ± 1.0°C ). Light levels were measured every other week during the study using a LI-185A photometer (Li-Cor, Lincoln, NE).

### Histology:

Eight seedlings were harvested every five days from days 5 to 35. The hypocotyl region was collected from seedlings on all days and cotyledons were collected from seedling harvested on days 5, 10 and 15. Tissues were fixed using FAA (Formalin-Acetic acid-Alcohol with10 mL formalin [37% formaldehyde], 5 mL glacial acetic acid, 50 mL 95% ethanol and 35 mL deionized water), dehydrated

in tertiary butyl alcohol and embedded in melted paraplast (Monoject Scientific, St. Louis, MO) using standard methods of microtechnique (Sass 1958; Ruzin 1999). The paraffin-embedded tissues were microtomed (American Optical Instruments Company, model 820, Buffalo NY) both transversely and longitudinally to obtain 10 μm thick serial sections. Tissues then were stained using Johansen's safranin and fast green protocol (Johansen 1940; Ruzin 1999). Permanent slides were made of the serial sections, and studied using a light microscope. Digital photographs were taken of representative seedlings in successive stages of development using an Olympus BX50 microscope.

## RESULTS

The morphology of *Stylisma pickeringii* var. *pattersonii* seedlings proceed though four distinct stages of development (Figure 6). Initially, the radicle emerges from the seed coat 5 days after planting. Following the emergence of the radicle, an enlarged ring, referred to as the transition zone, develops below the surface of the media. The transition zone is outwardly visible as early as 12 days after planting the seed. A dark purple region, corresponding to the upper portion of the fused cotyledonary petioles, elongates above the transition zone and below the free distal portion of the cotyledons. A lateral shoot then emerges from the transition zone 21 days after planting the seed.

Longitudinal sections of the basal portion of the free cotyledons of *Stylisma pickeringii* seedlings indicate that the shoot apical meristem is not present at the end of an elongating stem, emerging between the cotyledons, as typical in many seedlings. Instead the apical meristem occurs in a cavity created by the fusion of the cotyledonary petioles (Figure 7). This narrow cavity, measuring approximately 0.10 µm in diameter, extends from the basal region of the free cotyledons to the shoot apical meristem, below the soil surface (Figure 8). The shoot apical meristem is located at the base of the cavity (Figure 7). The presence of a shoot apical meristem was documented within the cavity on the same day as radicle emergence from the seed coat, five days after planting (Figures 7 and 9).

Due to cell division at the shoot apical meristem, the region enlarges forming a transition zone that is evident at the base of the fused petiole, outwardly appearing to be the hypocotyl (Figure 6). In selected serial cross sections advancing from the apex of the fused cotyledonary petioles to the hypocotyl, an increase in diameter is visually evident at the base of the fused petioles where the shoot apical meristem is situated (Figure 8).

Leaf primordia are present as early as day 15, which is prior to the emergence of the lateral shoot, occurring as early as day 25 (Figure 9). The lateral shoot grows in diameter while elongating within the cavity until it reaches a size that is too large to be contained by the cavity. The lateral shoot then emerges as the fused cotyledonary petioles separate (Figures 9 and 10).

## DISCUSSION

*Stylisma pickeringii* var. *pattersonii* seedlings have an unusual development at the macroscopic level; however, anatomical examination of the seedlings indicates development is quite similar to typical seedling development. *Stylisma pickeringii* seedlings have a shoot apical meristem situated between the cotyledons, except the petioles of the cotyledons are fused below the soil surface which gives the appearance of a lateral shoot arising from the hypocotyl region. The transition zone, seen prior to lateral shoot emergence, is simply a result of cell division at the shoot apical meristem applying pressure to expand the petioles that surround it. Once the shoot apical meristem is too large to be contained within the cavity created by the fusion of the cotyledonary petioles, a lateral shoot becomes outwardly visible as it ruptures the fused petioles that encircle it.

Sand habitats frequently have shifting sands, an upper soil profile that lacks moisture, high temperatures and high light intensities that make seedling establishment difficult (Bach, 1998). The fusion of the cotyledonary petioles may enhance the ability of *Stylisma pickeringii* seedlings to become established in this harsh environment. The lateral shoot, developing within the protective sheath formed by the cotyledonary petioles, would be shielded from blowing sand in contrast to new shoot developing above the soil surface. This protection would reduce the chance of the shoot apical meristem being physically damaged by

blowing sand. Decreased exposure to wind and high light intensities may also decrease transpiration rates in the young lateral shoot. Increased humidity levels within the cavity created by the fused cotyledonary petioles may also decrease evaporation, placing less water stress on the developing lateral shoot and enhancing its ability to become established. As the lateral shoot grows through the cavity created at the center of the fused cotyledonary petioles, there is more time for the roots to develop and become established before the lateral shoot emerges through the soil surface, decreasing the chance of desiccation. This adaptation would be very beneficial to a seedling that must become established in a hot and dry environment such as found in sand prairies. Future studies should examine other sand prairie species to determine if similar adaptations have arisen.

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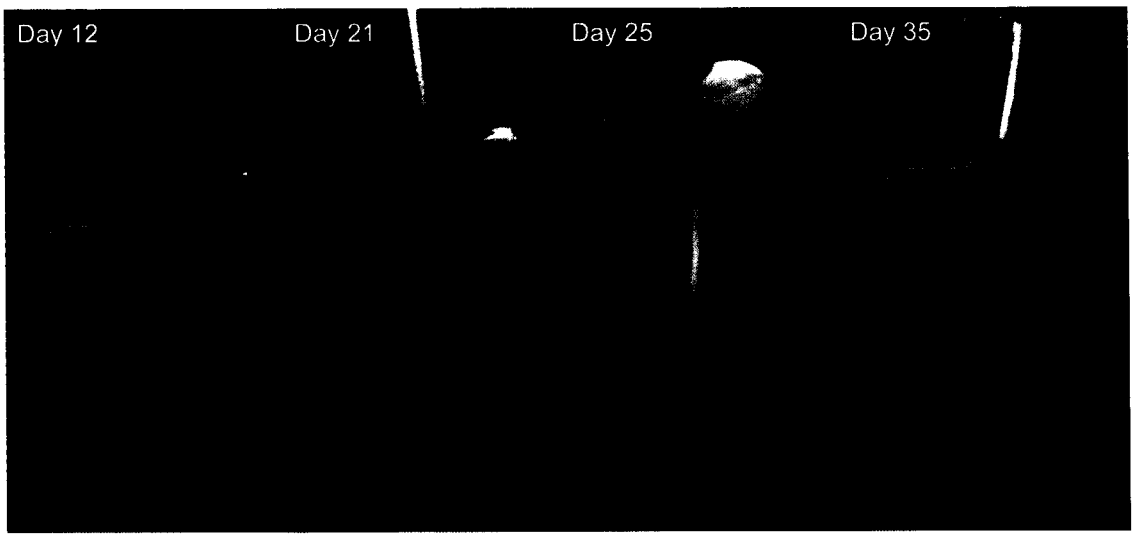



Figure 6. Successive stages in the external development for seedlings of *Stylisma pickeringii* var. *pattersonii* grown *in vitro* (R, Radicle; Tz, Transition Zone; Sc, Seed Coat; Fc, Fused Cotyledonary Petioles; Ls, Lateral shoot)

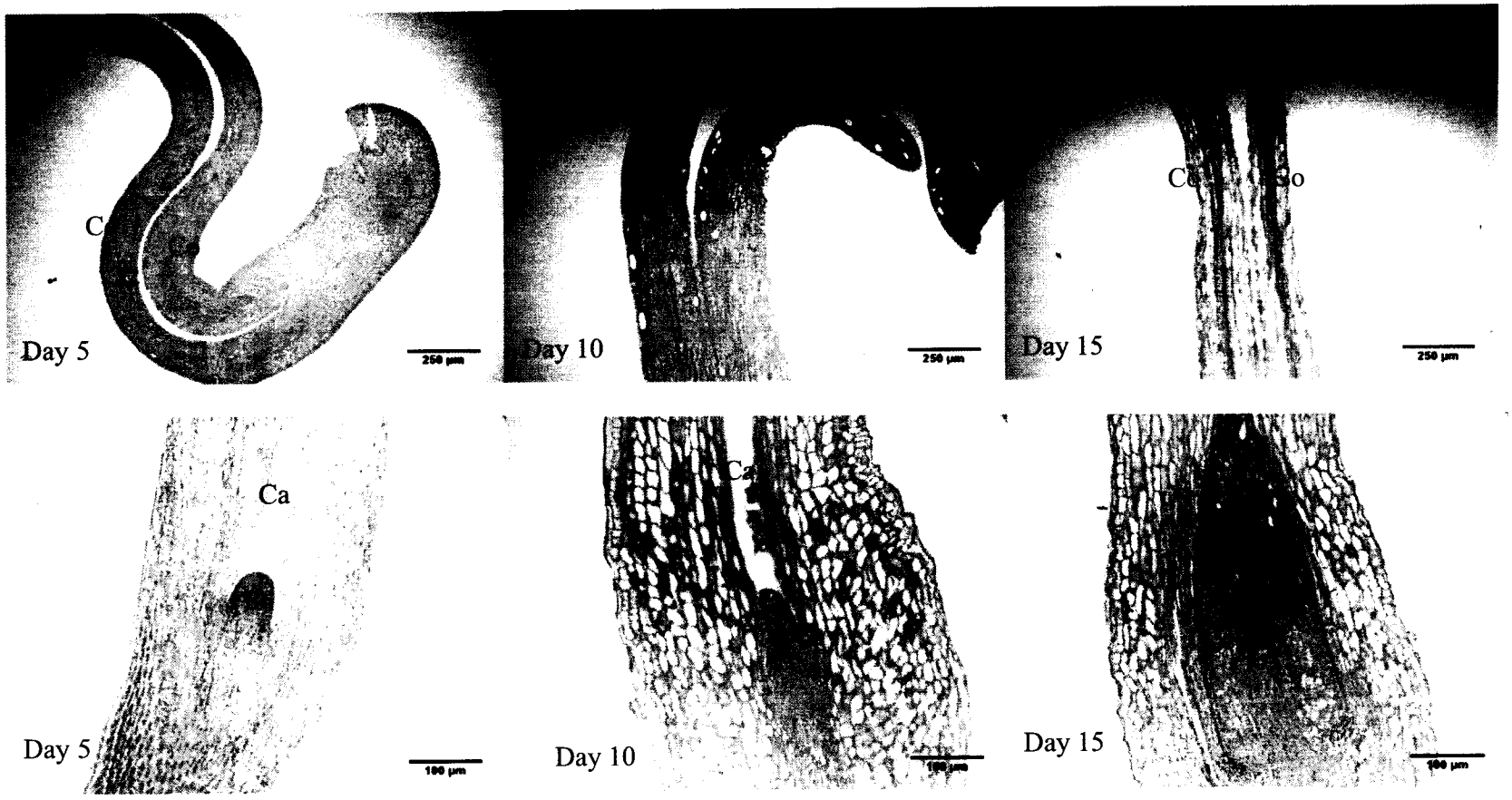
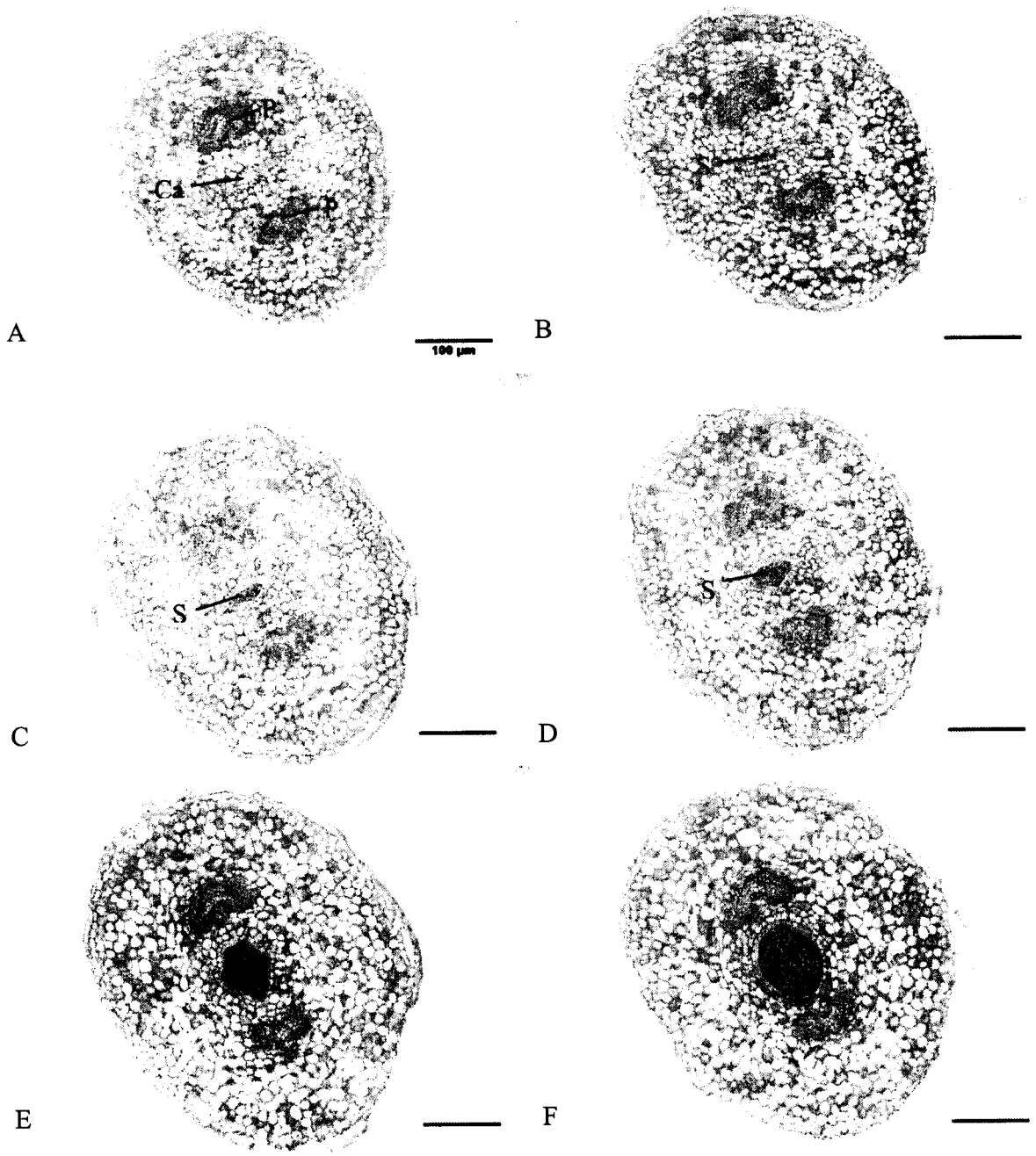



Figure 7. Selected longitudinal sections of the cotyledons (upper row) and apical meristem, encapsulated within the fused cotyledonary petioles (lower row) of *Stylisma pickeringii* var. *pattersonii* at successive stages of seedling development on days 5, 10 and 15 after imbibition (Co, Cotyledon; S, Shoot Apical Meristem; Ca, Cavity).




A B C D E F

Figure 8. Selected serial cross sections of the apical meristem, encapsulated within the fused cotyledonary petioles of *Stylisma pickeringii* var. *pattersonii* after 15 days of development, passing proximally toward the radicle from A to F (Ca, Cavity; S, Shoot Apical Meristem; P, Procambial Strands).

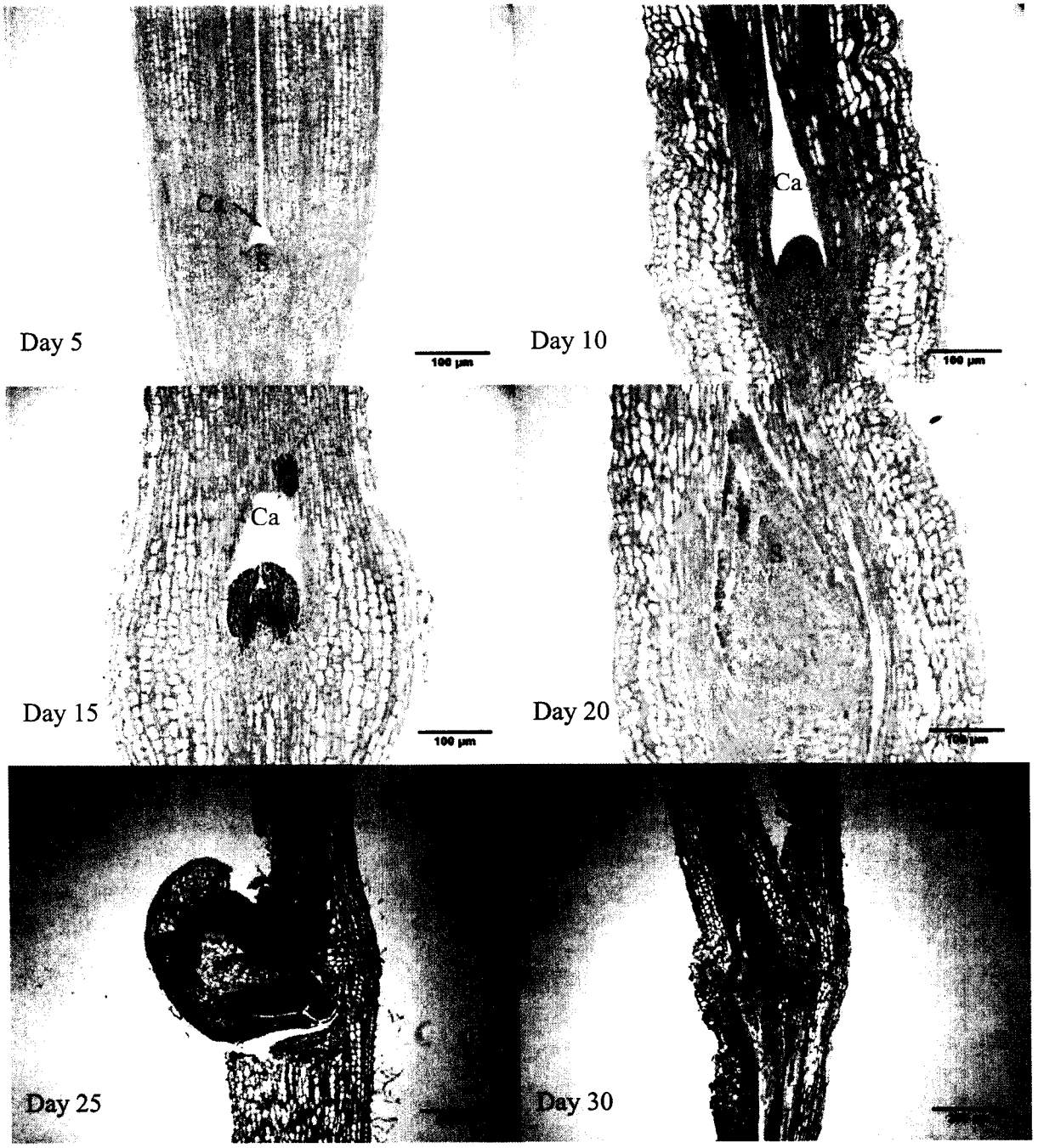



Figure 9. Selected longitudinal sections of the apical meristem and lateral shoot of *Stylisma pickeringii* var. *pattersonii* at successive stages of seedling development on days 5, 10, 15, 20, 25 and 30 after imbibition (Ca, Cavity; S, Shoot Apical Meristem; Ls, Lateral Shoot).

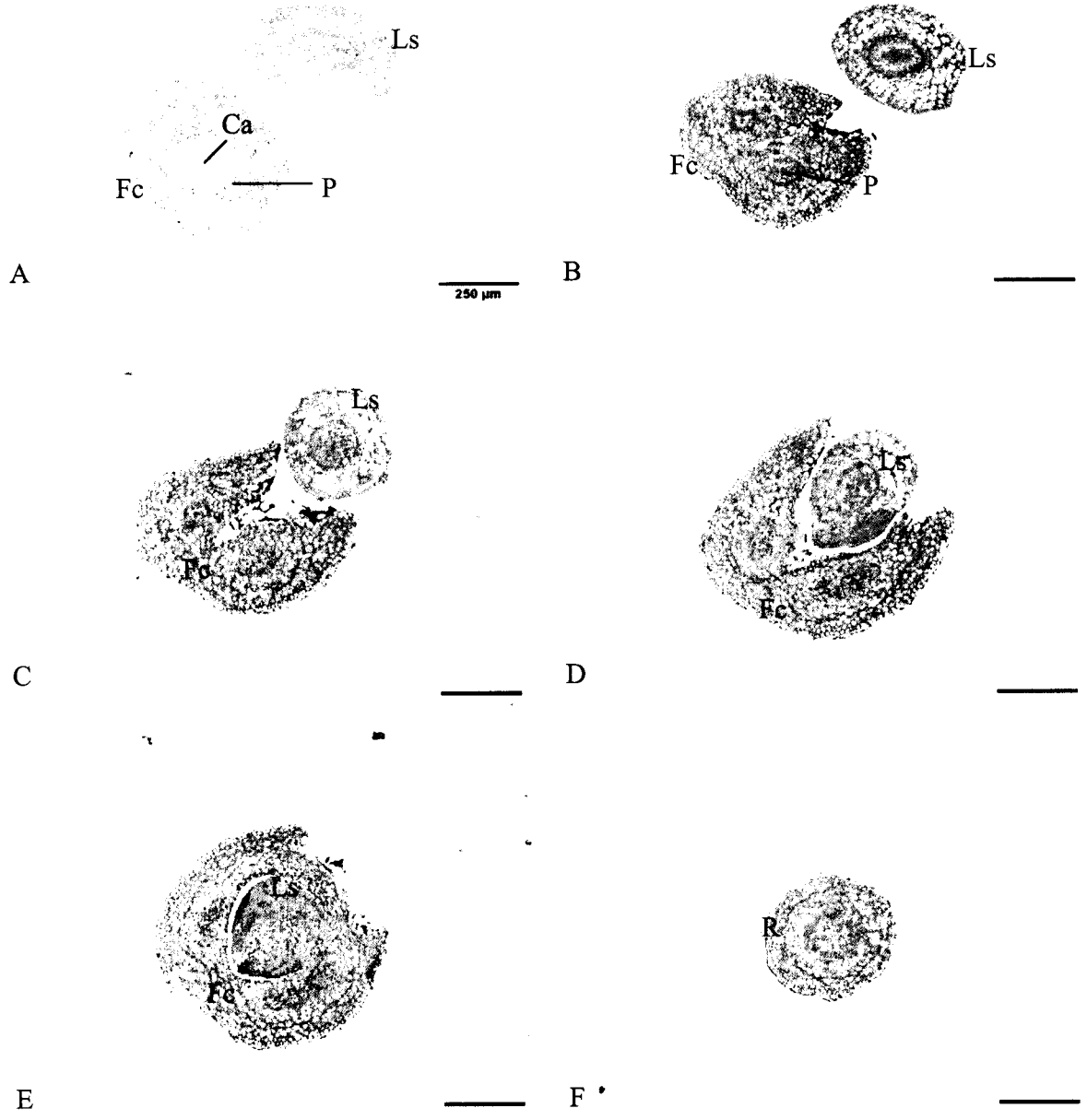



Figure 10. Selected serial cross sections of the apical meristem and lateral shoot of *Stylisma pickeringii* var. *pattersonii* after 30 days of development, passing proximally toward the radicle from A to F (Ca, Cavity; Fc, Fused Cotyledonary Petioles; P, Procambial Strands; Ls, Lateral Shoot; R, Root).

# CHAPTER 4:

# Conclusions

The preceding studies have examined seedlings of *Stylisma pickeringii* var. *pattersonii*, an endangered plant in Illinois. The primary objectives of these studies were to determine the role of light in *S. pickeringii* seedling development and to document the anatomical development of *S. picker*ingii seedlings.

While previous studies demonstrated that light was essential for lateral shoot development, this study indicated that total irradiance rather than photoperiod had a more significant role in seedling development. The initiation of radicles, transition zones, and cotyledons all occurred earlier with higher light intensities than with lower light intensities. Lateral shoot initiation occurred earlier with long days than with short days, and within short days lateral shoots of higher light intensity plants emerged earlier than those of lower light intensity. Although the initiation of distinct stages in seedling development was accelerated by higher light, growth following initiation was greater at lower light intensities. This finding was very intriguing since *Stylisma pickeringii* grows in habitats that typically have light intensities that exceed those used in this study. Plants grown in tissue culture typically have a thinner cuticle and fewer functioning stomates, which could cause them to lose more water under higher temperature conditions which might be created with higher light. Increased transpiration rates caused by higher light conditions and perhaps higher leaf temperatures may be a cause for

the decreased growth rates of seedlings grown with higher light conditions. Future studies determining the optimal light conditions for lateral shoot growth subsequent to lateral shoot initiation would assist in developing management strategies for this Illinois endangered species. Further examination of factors influencing *S. pickeringii* seedling development in soil, such as how light intensity relates to growth rates of the lateral shoot, would greatly enhance our understanding of how this plant interacts with its environment.

Seedlings of *S. pickeringii* grown in tissue culture differ from those in the field. Seedlings in the field and even those grown in soilless mix had several lateral shoots originating from one location; however, no seedlings grown in these tissue culture developed more than one lateral shoot from a single transition zone. Future studies should examine the differences between seedlings grown *in vitro* and those grown in soil and attempt to determine possible causes of this variation.

*Stylisma pickeringii* var. *pattersonii* outwardly appears to have a very unusual pattern of seedling development. The anatomical investigation of these seedlings indicated that *S. pickeringii* seedlings have fused cotyledonary petioles that encase the shoot apical meristem in a cavity below the soil surface. This adaptation enables the young shoot to mature within the protected sheath before it emerges into the harsh sand prairie environment. While the lateral shoot is a distinctive characteristic of *Stylisma pickeringii* seedlings, the fused cotyledonary

petioles and its sunken shoot apical meristem may be the most significant factors enhancing seedlings establishment. Although this adaptation appears to be unique to *S. pickeringii*, future morphological and anatomical studies of sand prairie plants may show that this adaptation to the harsh environment occurs in several other species.

*Stylisma pickeringii* var. *pattersonii* is certainly a fascinating product of the unique sand prairie environment. Blowing sand, low levels of moisture, high light intensities, and high temperatures, all common in the sand prairie, have led to the adaptations present in this species. This study serves to improve our understanding of how *Stylisma pickeringii* seedlings become established and the close relationship between these plants and their environment.

## APPENDIX A:

## Plant Microtechnique Checklist

___ 1. Place specimen in FAA (Formalin-Acetic acid-Alcohol with10 mL formalin [37% formaldehyde], 5 mL glacial acetic acid, 50 mL 95% ethanol and 35 mL deionized water), let sit for at least 24 hrs, label (species, solution, etc.)

___ 2. Decant FAA, refill with 70% Ethanol, place on labeled notecard, let sit for at least 24 hrs

___ 3. Decant 70% Ethanol, refill with fresh 70% Ethanol, place on labeled notecard, let sit at least 24 hrs

___ 4. Decant 70% Ethanol, refill with 100% Ethanol, place on labeled notecard, let sit at least 24 hrs

___ 5. Decant 100% Ethanol, refill with 50 parts Absolute Ethanol: 50 parts TBA (Tertiary Butyl Alcohol), place on labeled notecard, let sit for at least 2 hrs

___ 6. Decant 50: 50 mix, refill with 25 pts Absolute Ethanol: 75 pts TBA, place on labeled notecard, let sit at least 2 hrs

* steps 7 – 10 must be performed on the same day

___ 7. Decant 25: 75 mix, refill with 100% TBA and a small pinch of Eosin stain, place on labeled notecard, let sit for 3 hours minimum

___ 8. Decant 100% TBA and Eosin and repeat step 10

___ 9. Decant 100% TBA and Eosin and repeat step 10

___ 10. Pour out half of the 100% TBA and Eosin, pour melted paraffin into jar to create 50 parts TBA: 50 parts paraffin solution and place this container into the paraffin oven for at least 2 hrs but this may remain in the oven overnight (oven should be set at the melting point of the paraffin)

* steps 11 – 13 must be done in the paraffin oven
(not allowing the paraffin to harden)

___ 11. Decant 50: 50 mix into paraffin waste container, refill with 100% melted paraffin and let it sit at least 2 hrs in the oven

___ 12. Decant the pure paraffin into the waste container quickly, refill with 100% melted paraffin and let it sit at least 2 hrs in the oven

___ 13. Decant the pure paraffin into the waste container quickly, refill with 100% melted paraffin and let it sit at least 2 hrs in the oven

___ 14. Make a paper boat using a note card (see Sass, 1958)

___ 15. Quickly pour pure melted paraffin and tissue into the paper boat and top off the boat with melted paraffin (this must be done on a slide warmer)

___ 16. Reposition the tissue so that it is in approximately the correct place and place a label on the edge of the block with writing (in pencil) facing out

___ 17. When crystals begin to form at surface of the paraffin use a dissecting needle to position the tissue into the desired position. Try to lift the tissue from the bottom of the boat so it is completely covered by paraffin. If the paraffin becomes to stiff place the boat back onto the slide warmer to melt the paraffin

___ 18. When the paraffin becomes pudding-like, place the boat in an ice slurry and allow it to harden, it may take approximately an hour

___ 19. Remove the boat from the ice water and allow the paper to air dry

___ 20. Peel the paper from the paraffin block

___ 21. Heat a non-disposable scalpel using an alcohol lamp

___ 22. Use the hot scalpel to cut/melt the paraffin block, separating the specimen but leaving plenty of wax around it

___ 23. Use a razor blade to shave paraffin and shape the block containing the specimen leaving the base wider than the top

___ 24. Using the razor blade make two sides of the face of the block parallel to one another (this may need to be adjusted once sectioning begins to create a straight ribbon)

___ 25. Drip paraffin onto wood block covering the top of the block and about ¼" down each side

___ 26. Adhere the label for the specimen on the wood block using melted paraffin to adhere the paper to the block and cover it with paraffin as well

___ 27. Warm the scalpel blade and melt some paraffin on the top of the block, place the paraffin block with specimen in it on top of the wood block

___ 28. Drip paraffin around all the edges of the paraffin block containing the specimen

___ 29. Place wood block in refrigerator for at least an hour (to ensure the paraffin has hardened)

___ 30. Secure the blade in the microtome and place the wood block in the clamp of the microtome

___ 31. Position the blade so that it is very close to the paraffin block

___ 32. Make sections (7-9 µm)

___ 33. Use a small paintbrush to hold the ribbon, do not touch the ribbon to your skin (the heat from your skin will melt the paraffin to you)

___ 34. Place ribbon on a piece of paper in a transparency sheet box, the ribbon should be placed with the shiny side down, label appropriately

___ 35. Soak "pre-cleaned" slides in a jar with 70% Ethanol and 2-3 drops of 1M HCl for at least 24 hrs

___ 36. Place 1-2 drops of Haupt's adhesive (1.0 g Knox unflavored gelatin, 2.0 g Phenol crystals, 15.0 ml Glycerine, and 100.0 ml of distilled water [30°C ], then filter this solution through a cheesecloth) on a slide a spread it around with a gloved hand until it covers about 7/8 of the slide and has reached a clear and tacky stage, place on a slide warmer (36-37°C), cover the slide warmer and let them sit overnight

___ 37. Remove the slide from the warmer and use a dropper to squirt a 1% formalin solution onto the slide until a pool of it covers the Haupts adhesive

___ 38. Cut the ribbon into smaller pieces (~2") with a razor blade

___ 39. Move the smaller pieces of the ribbon onto the slide and float them on top of the 1% formalin pool

___ 40. Place the slide onto the slide warmer (36-37°C) for about one hour, if the pool begins to bubble reduce the heat of the warmer

___ 41. Move the ribbons with a dissecting needle so that they are not touching one another

___ 42. Turn the slide warmer off and cover it, allow them to sit overnight so the formalin pool evaporates

___ 43. Store slides in dust free container (if storing long-term be sure to store them on their back, the ribbons may shift if on their side)

## APPENDIX B:

### Staining Procedure (Safranin O and Fast Green)

___ 1. Arrange Coplin staining jars as below and label them appropriately

Xylene I → Xylene I I→ 100% EtOH → 95% EtOH → 70% EtOH → 50% EtOH
↓
Safranin O
↓
Tap Water
↓
Xylene II ← Xylene I ← 100% EtOH ← 95% EtOH ← 70% EtOH ← 50% EtOH

___ 2. Remove slide with ribbons fixed to it from dust-free storage using forceps

___ 3. Place slides in Xylene I Coplin jar and agitate for approximately 1 min. (this step remove the paraffin from the slide)

___ 4. Remove slides from Xylene I, drain excess solution by touching edge of slide to a clean paper towel

___ 5. Place slides in Xylene II Coplin jar for 1 min. (removes residual paraffin)

___ 6. Remove slides from Xylene II, drain excess solution

___7. Place slides in 100% EtOH for 10 to 20 sec. with agitation

___ 8. Remove slides from 100% EtOH, drain excess solution

___ 9. Place slides in 95% EtOH for 10 to 20 sec. with agitaion

___ 10. Remove slides from 95% EtOH, drain excess solution

___ 11. Place slides in 70% EtOH for 10 to 20 sec. with agitaion

___ 12. Remove slides from 70% EtOH, drain excess solution

___ 13. Place slides in 50% EtOH for 10 to 20 sec. with agitaion

___ 14. Remove slides from 50% EtOH, drain excess solution

___ 15. Place slides in Safranin O for 2 to 24 hours
Check slides for time by rinsing in tap water and viewing under scope
(Safranin should appear bright red in nuclei, chromosomes and lignified cell walls)

___ 16. Remove slides from Safranin, drain excess stain

___ 17. Wash off excess stain by dipping the slide in beaker full of tap water, drain excess water

___ 18. Place slide in 50% EtOH for 10 sec. (longer if de-staining which means that the safranin was to strong and needs to be washed out)

___ 19. Remove slides from 50% EtOH, drain excess solution

___ 20. Place slide in 70% EtOH for 10 sec.

___ 21. Remove slides from 70% EtOH, drain excess solution

___ 22. Place slide in 95% EtOH for 10 sec.

___ 23. Remove slides from 95% EtOH, drain excess solution

___ 24. Place slide in 100% EtOH for 10 sec.

___ 25. Remove slides from 100% EtOH, drain excess solution

___ 26. Hold the slide with one hand and flood the slide with fast green stain from a dropper bottle

___ 27. Tip the slide to distribute the stain evenly over all sections

___ 28. After 5 to 30 sec. (depending on the strength of the stain) drain the fast green into another dropper bottle marked "Used Fast Green" (this can be re-used later)

___ 29. Immediately flood the slide with pure Clove oil and tip to distribute it

___ 30. Drain the Clove oil into the "Used Fast Green" bottle

___ 31. Place the slide in Xylene I Coplin jar for 1 min.

___ 32. Remove the slides from Xylene I, drain excess solution

___ 33. Place the slide in Xylene II Coplin jar for at least 1 min. but they may remain there until ready to mount coverslip

## APPENDIX C:

### Equipment, Materials and Solutions Frequently Used in Histology

Equipment

Microtome – instrument used for sectioning very thin slices of tissue for microscopic examination
Paraffin oven – warming oven capable of maintaining a constant temperature often near 58°C
Slide warmer – used to adhere sections, paraffin ribbons, to slide in making permanent slides (temperature maintained at 38 – 40°C)
Embedding plate – similar to slide warmer, used to keep paraffin molten while embedding tissue (temperature maintained at 55°C)

Materials

Small vials – used to store tissue during killing and dehydration must have cap that can seal to reduce evaporation
Paraffin – wax used to embed tissue prior to sectioning commonly Paraplast is used
Notecard – used to make disposable tray/boat for embedding tissue
Dissecting Needles – used to manipulate tissue during the embedding process
Non-Disposable Scalpel – used to melt paraffin and shape blocks
Mounting Blocks – used to mount paraffin embedded tissue for sectioning these may be made from wood scraps or purchased
Disposable Microtome Knives – these knives are very convenient because they do not require shapening
Small Paint Brush – used to handle paraffin ribbon during sectioning No. 1 or 2 camel hair brushes are preferred
Empty Transparency Box – used to store ribbons before mounting them on slides any thin box with a lid will serve the same purpose
Diamond Pen – used to etch labels on slides before staining
Coplin Jars – jars used in staining process by holding slides upright at least 13 Coplin jars are needed (one for each step in staining)
Dropper Bottles – used during staining process must have rubber stopper to prevent evaporation
Forceps – used to move slides from one Coplin jar to another during staining heavy forceps with a straight point are preferred
Slides – 1" x 3" are commonly used
Paperclips – used to hold coverslip on the slide while mounting with Permount
Slide Boxes – used to store either permanent slides or slides prior to staining

Solutions

Formalin-Acetic acid-Alcohol (FAA) - 10 mL formalin [37% formaldehyde]
5 mL glacial acetic acid
50 mL 95% ethanol
35 mL deionized water
- used to fix and kill cells

Ethanol – used in dehydration series and staining
several percentages used including 100%, 95%, 70% and 50%

Tertiary Butyl Alcohol (TBA) – dehydration agent used in preparation for paraffin infiltration
- TBA and EoSin Y stain are used to make tissue visible in paraffin (only a pinch of EoSin Y is needed for 1L of TBA)

Haupt's Adhesive - 1.0 g Knox unflavored gelatin
2.0 g Phenol crystals
15.0 ml Glycerine
100.0 ml of distilled water [30°C ]
filter solution through a cheesecloth before using
- used to adhere paraffin sections to slide

1% Formalin – used to flood slide and expand sections when adhering paraffin sections to slide

Xylene – removes paraffin from sections in first steps of staining process
washes away excess fast green in last steps of staining

Safranin O – 2.0 g Safranin O powder
100 ml Methyl Cellulose
when in solution add the following:
50 ml 95% Ethanol
50 ml distilled water
2.0 g Sodium Acetate
4.0 ml Formalin
- used to stain lignified cell walls, nuclei and chromosomes

Fast Green – 1.0 g Fast Green powder
100 ml Methyl Cellulose
100 ml Pure Clove Oil
- used as background stain
may need diluted with extra clove oil

Pure Clove Oil – used to clear slide of fast green during staining

Permount – used to mount coverslip on permanent slide